BIOORGANIC CHEMISTRY 8, 299–309 (1979)

Some Novel Inhibitors of Porcine Pancreatic Elastase

CEDRIC H. HASSALL, WILLIAM H. JOHNSON, AND NOEL A. ROBERTS

Roche Products Limited, Welwyn Garden City, Herts, U.K.

Received January 29, 1979

New evidence has enabled the identification of novel specific inhibitors of porcine pancreatic elastase with potential for action *in vivo*. The basis for optimization of activity in this series of derivatives of alanylproline, and the relationship of elastase inhibition to the binding mode, is discussed.

Although the role of elastin in structural tissue is little understood, there is good evidence to suggest that destruction of this protein by specific proteases, the elastases, is an important factor in the tissue degradation associated with pancreatitis (*1*), arthritis (*2*), and emphysema (*3*, *4*). Consequently, specific inhibitors of elastases with reasonable survival times, *in vivo*, might have potential for the management of such disease states.

Various proteases of the elastase type have been identified. We have concentrated in this investigation on porcine pancreatic enzyme (EC 3.4.4.7), a form of elastase which has been the subject of extensive biochemical and structural studies. When this investigation was initiated some 6 years ago, there was already a basis for designing specific inhibitors of porcine pancreatic elastase. An elegant X-ray diffraction study of the tosyl-derivative at 3.5 Å (*5*) and a complete amino acid sequence analysis (*6*) had been completed. It was established that the active center and the mechanism of action on peptides had close similarities to the case of α-chymotrypsin and other serine proteases. Moreover, the specificity of reactions with particular small peptides had been related to the existence of two categories of enzyme binding subsites[1]; S_1–S_5 (with the corresponding substrate amino acid residues, P_1–P_5) are concerned with the *N*-terminal section, and S_1' and S_2' are associated with the *C*-terminal section of the amide being cleaved (*8–10*). As a result, a series of tri- and tetrapeptide chloromethyl ketones (*11*, *12*), and other derivatives (*13*, *14*) have been designed as active-center-directed irreversible inhibitors; other less specific inhibitors which reacted with the active-center serine-188 (cf. α-chymotrypsin, serine-195) have also been identified (*15–17*) but in no cases had these compounds shown prospects for therapeutic use in man. These and subsequent studies reported during the course of our investigation have, however, contributed important information concerning a further basis for selecting di-, tri-, and tetrapeptides (*18–21*) and related derivatives incorporating aldehyde (*22*, *23*), further chloromethyl ketone (*24*), trifluoroacetyl (*25*, *26*), acylcarbazate (*19*, *27*), and other functions (*28–30*) to serve as inhibitors of porcine pancreatic elastase. In what follows we shall describe studies leading to the identification of improved specific inhibitors with

[1] Nomenclature according to Schechter and Berger (*7*).

0045-2068/79/030299-11$02.00/0

potential for application *in vivo*. Only reversible inhibitors have been studied; we consider that compounds which rely for their action on combination with serine, histidine, or aspartic acid residues of the active center, even when site directed (*3*), have not yet shown sufficient promise as selective elastase inhibitors for therapeutic application.

RESULTS AND DISCUSSION

Structure–Activity Studies

In order to improve the prospects for the inhibitors surviving, *in vivo*, we have attempted to minimize the size of the peptides and to utilize functional groups less likely to be rapidly removed. Our initial studies concentrated on variations of the tripeptide derivatives Ac-Ala-Ala-Ala-OH (I) and Ac-Ala-Pro-Ala-OH (II). This choice was

TABLE 1

REPLACEMENT OF ALANINE BY AMIDES IN P_1

P_4 P_3 P_2 P_1		K_i (mM)
Ac·Ala·Ala·Ala·OH	(I)	1.6
Ac·Ala·Pro·Ala·OH	(II)	1.0
Ac·Ala·Ala·NHEt	(III)	1.45
Ac·Ala·Ala·NHiPr		1.5
Ac·Ala·Ala·NHsBu		1.8
Ac·Ala·Pro·NHEt		0.8

based on previous evidence of good binding (*8*, *9*, *31*); the *N*-terminal alanine residue (P_3) was known to be critical and has been retained throughout the series; this is further confirmed in these studies by the observation that replacement of P_3-alanine by proline and by glycine gave compounds which were inactive and 15-fold less active (Table 2), respectively. Similarly, studies by others have suggested that proline is preferred to alanine as the P_2-residue in inhibitors (*17*). Our studies indicate that P_2-Pro may have advantages in some cases but in others there is no significant difference (Table 2). We

TABLE 2

EFFECT OF CHANGES IN P_3 AND P_2

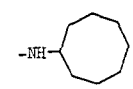
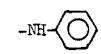
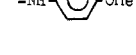
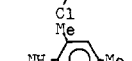
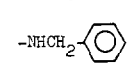
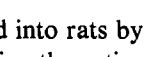
P_4 P_3 P_2 P_1	K_i (mM)
Pr·Ala·Ala·NHEt	0.2
Pr·Ala·Pro·NHEt	0.065
Pr·Ala·Ala·NHPh	0.040
Pr·Ala·Pro·NHPh	0.040
Z Ala·Ala·NHEt	0.4
Z Ala·Pro·NHEt	0.2
Z Gly·Pro·NHEt	3
Z Pro·Pro·NHEt	Inactive

favor proline because compounds derived from Ala · Pro are likely to be more stable *in vivo* to peptide hydrolysis than those based on Ala · Ala.

Variations in the residue, P_1. The fit of models of the acyltripeptides I, II to the shallow trough of the binding site was examined in a Watson–Kendrew-type skeletal model of porcine pancreatic elastase, which we constructed in accordance with the findings of Watson, Shotton and co-workers (*5*). This suggested that the corresponding *N*-alkyl-dipeptides lacking the P_1-Ala residue should bind in a manner similar to tripeptides with *C*-terminal alanine; evidently such dipeptides would have the advantage of being unaffected by the action of carboxypeptidases *in vivo*. The comparison of the K_i values of the Ac-Ala-Ala-Ala-OH (I) and Ac-Ala-Ala-NHEt (III) confirmed this (Table 1).

TABLE 3

Changes in P_1 with Pr.Ala.Pro as $P_4P_3P_2$

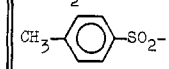
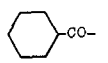
P_1	K_i (mM)	P_1	K_i (mM)
-NHCH$_3$	1.8		0.035
-NHCH$_2$CH$_3$ (IV)	0.065		0.040
-NHCH(CH$_3$)$_2$	0.45		0.060
-NH(CH$_2$)$_4$CH$_3$	0.23		0.11
 (V)	0.040		INACTIVE
	0.020		1.2
	0.025		

When this derivative (III) was introduced into rats by the oral route it was found, in repeat experiments, to be effective in inhibiting the action of porcine pancreatic elastase in inducing paw edema; evidently the compound (III) was sufficiently well absorbed and sufficiently stable *in vivo*, under these conditions.

Further alternatives to *N*-ethyl-P_1 functions have been investigated (Table 3). This has established that some improvement in inhibition could be achieved by replacing ethyl by cyclo-alkyl or aryl groups but, in the latter case, benzyl or heavily substituted phenyl groups were not favored.

Variations in the residue P_4. The effect of changing the residue P_4 in the sequence P_4-Ala · Pro · NHEt is set out in Table 4. Propionyl is preferable to acetyl in this position but the higher alkyl analogs were not remarkably dissimilar to propionyl. Nevertheless, cyclo-alkanoyl and benzoyl as P_4-residues increased the degree of inhibition to a level similar to that of the compound Ac-Pro-Ala-Pro-NHEt with P_5 and P_4 binding functions. It was found that *D*-alanine could not be substituted for P_4-proline. When

TABLE 4

Changes in P_4 with Ala.Pro.NHEt as $P_3P_2P_1$			
P_4	K_i (mM)	P_4	K_i (mM)
CH_3CO-	0.8	PhCO-	0.026
CH_3CH_2CO-	0.065	$Ph.CH_2CO$-	0.086
$(CH_3)_2CHCO$-	0.06	$Ph.CH_2OCO$-	0.20
$(CH_3)_3C.CO$-	0.08		1.35
$CH_3(CH_2)_3CO$-	0.08	Ac.Pro-	0.02
CH_3CH_2OCO-	0.15	Ac.D.Ala-	INACTIVE
	0.01		
Changes in P_4 with Ala.Pro.NHPh as $P_3P_2P_1$			
P_4	K_i (mM)	P_4	K_i (mM)
CH_3CO-	0.086	CF_3CO-	0.0013

trifluoroacetyl replaces acetyl as the P_4-residue, there was a substantial increase in inhibitory activity, as already observed by Dimicoli *et al.* (*25*, *26*), for related compounds (Table 4).

Relationship of P_1, P_4 residues. The results in Tables 3 and 4 provide a basis for optimizing activities associated with the interactions P_1–S_1 and P_4–S_4, independently. It was anticipated that a compound with each of the residues at P_1, P_4 favored for activity, independently, should be a potent inhibitor. This did not prove to be the case. Compounds with both P_1 and P_4 as alicyclic or aromatic residues were inactive; any combination with both P_4 larger than propionyl and P_1 larger than ethylamide had decreased activity (Table 5). Moreover, in contrast to the case of propionyl-Ala-Pro-P_1 (Table 3) increasing the size of P_1 in compounds with the general formula benzyloxycarbonyl-Ala-Pro-P_1 reduced the activity of inhibitors (Table 5). We attribute these nonadditive effects to differences in the binding mode for compounds optimized,

TABLE 5

Effect of changes in P_1 and P_4	
P_4 P_3 P_2 P_1	K_i (mM)
PhCO-Ala.Pro.NHPh	INACTIVE
$CH_3(CH_2)_4CO$-Ala.Pro.NHPh	INACTIVE
Z-Ala.Pro.$NHCH_2CH_3$	0.2
Z-Ala.Pro.$NHCH(CH_3)_2$	1.3
Z-Ala.Pro.NH 	1.7
Z-Ala.Pro.$NH(CH_2)_4CH_3$	>5
Z-Ala.Pro.NHPh	INACTIVE

separately, for binding at S_1 and S_4 sites. Some evidence bearing on this has come from X-ray diffraction studies.

X-Ray Diffraction Studies

Earlier X-ray diffraction studies, at 3.5 Å resolution (*5*), of the binding of free di-, tri-, and tetrapeptides to the active site of porcine pancreatic elastase have indicated, unexpectedly, that the binding mode on the amino-terminal end differed from that observed for trypsin and α- and γ-chymotrypsins; in each of the latter cases substrate residues P_1, P_2, and P_3 appear to be associated with enzyme residues 214, 215, and

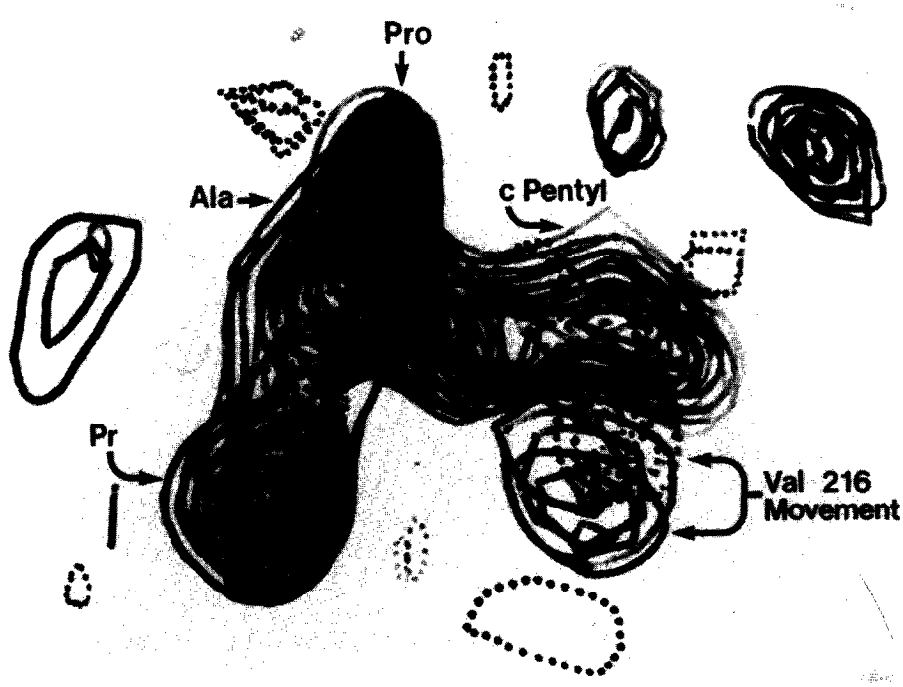


FIG. 1. Photograph of the differences in electron density between pr · Ala · Pro · NHcPentyl-elastase and tosyl elastase. The associated positive and negative peaks indicate a movement of Val-216.

216. In elastase, the similar substrate residues are oriented very differently in the direction of the main chain atoms of Thr-41 and Gln-192. Solution studies support this interpretation (*9*, *10*). Moreover, in elastase, the S_1-site is less critical to the binding than in related enzymes; the combined effects of up to eight subsites (*32*) determine the efficient binding of natural peptide substrates.

We have had the benefit of an X-ray diffraction study at 3.5 Å by Sawyer and Watson (*39*) on the two typical inhibitors, Pr · Ala · Pro · NHEt (IV) and Pr · Ala · Pro · NH · cPentyl (V), prepared in this investigation. This establishes that the binding mode of these inhibitors is along the chain in the vicinity of residues 214–216,[2] with the *C*-terminal groups lying between Phe-215 and Gln-192, and the *N*-terminal groups in the region between Trp-172 and Thr-179 (Figs. 1 and 2). Clearly, this

[2] The numbering of residues is in accord with Sawyer *et al.* (*33*).

orientation of the inhibitors differs from those observed by Shotton and co-workers for the inhibitors H · Ala · Ala · Ala · OH and Ac · Pro · Ala · Pro · Ala · OH (*31*). However, there was some suggestion in the studies by these investigators that for the inhibitor Ac · Ala · Ala · OH there was an additional, but at that time undefined, binding mode. It has been suggested (34) that for Ac · Ala · Pro · P_1 there is a different mode in which the carbonyl group of P_1 and the α-amino group of the P_4-residue were important. Assuming that the cases of the inhibitors IV, V and the substrates

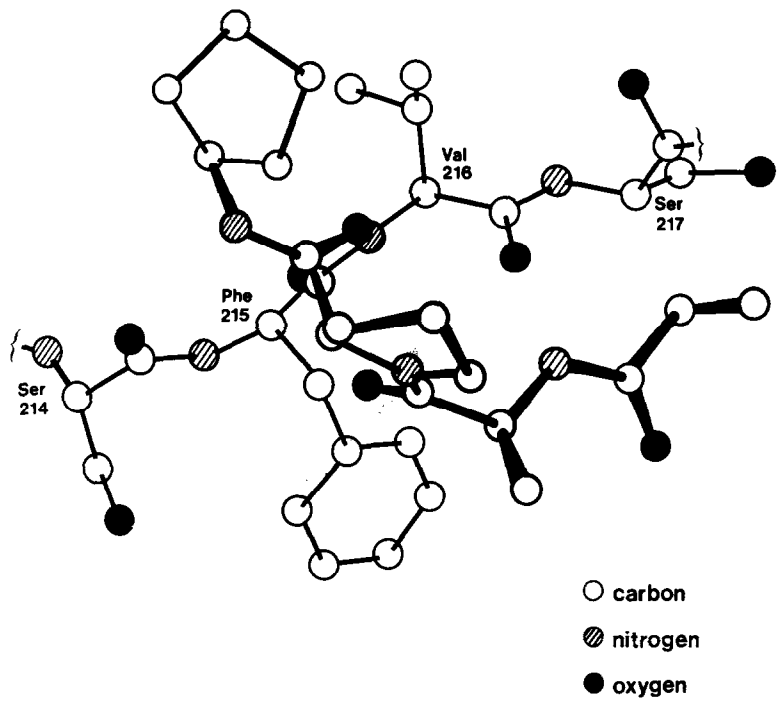


FIG. 2. Diagram showing the approximate mode of binding of Pr · Ala · Pro · NHcPentyl to elastase in the area of residues 214–217.

Ac · Ala · Pro · P_1 are related, the new X-ray diffraction studies on these inhibitors make it unlikely that these features of the P_1 and P_4 residues are determining.

CONCLUSIONS

These studies have resulted in the identification of particular simple dipeptide derivatives which inhibit porcine pancreatic elastase at low concentration (K_is 10^{-5}–10^{-6} *M*) and have good prospects for survival for sufficient periods to inhibit elastase, *in vivo*; this has been confirmed in the case of the typical compound Ac · Ala · Ala · NHEt by inhibition experiments in rats.

The structure–activity relationships, combined with X-ray diffraction data on the compounds Pr · Ala · Pro · NHEt (IV) and Pr · Ala · Pro · NH · cPent (V), have provided new evidence concerning the binding modes of such small peptide inhibitors.

EXPERIMENTAL

Synthesis

Mp's were determined with a Tottoli-type apparatus. Optical rotation measurements were with a Perkin–Elmer 141MC automatic polarimeter. All products and intermediates were homogeneous on t.l.c. and had the expected ^{1}H nmr spectra and microanalyses.

N-Benzyloxycarbonyl-L-alanyl-L-proline

A solution of proline (104 g, 0.903 *M*) in water (1350 ml) and triethylamine (267 ml, 1.8 *M*) was added to *N*-benzyloxycarbonyl-L-alanine-*N*-hydroxysuccinimide ester (289 g, 0.903 *M*) in 1:2-dimethoxyethane (1800 ml). The mixture was stirred (16 hr) and the 1:2-dimethoxyethane removed by evaporation. The aqueous solution, after extraction with ethyl acetate (2 × 300 ml) was acidified to pH 1–2 with hydrochloric acid. The oil was dissolved in ethyl acetate (2 × 900 ml), washed with water (2 × 400 ml), dried (Na_2SO_4), and evaporated to an oil. The product crystallized on the addition of diethyl ether (221.4 g, 77%), mp 124–125°C [lit (*35*) 120–122°C], $[\alpha]_D^{20} = -91.2°$ (*c* 1, MeOH).

Anal. for $C_{16}H_{20}O_5N_2$: C, 60.0; H, 6.3; N, 8.7. Found: C, 59.9; H, 6.4; N, 8.85%.

N-Benzyloxycarbonyl-L-alanyl-L-proline Anilide

N-Benzyloxycarbonyl-L-alanyl-L-proline (32 g, 0.1 *M*) in dry tetrahydrofuran (300 ml) was cooled to −10°C. *N*-Ethylmorpholine (12.7 ml, 0.1 *M*) and *iso*butylchloroformate (13.1 ml, 0.1 *M*) were added and the mixture stirred for 20 min at −10°C. Aniline (9.3 ml, 0.1 *M*) was added, the mixture stirred at 0°C (1 hr), room temperature (16 hr), and evaporated to a solid. Treatment with ether gave the product (33.2 g, 81%), mp 139.5–140.5°C, $[\alpha]_D^{20} = -125.5°$ (*c* 1, MeOH).

Anal. for $C_{22}H_{25}O_4N_3$: C, 66.8; H, 6.4; N, 10.6. Found: C, 66.6; H, 6.5; N, 10.4%.

N-Propionyl-L-alanyl-L-proline Anilide

The benzyloxycarbonyl compound (8.7 g, 0.022 *M*) was dissolved in 4 *N* hydrogen bromide in acetic acid solution (40 ml) and after 1 hr dry ether was added. The solid was filtered, washed with ether, dried, and dissolved in pyridine (120 ml). Propionic anhydride (5.73 ml, 0.044 *M*) was added and the mixture was stirred at room temperature (1 hr) and evaporated. The residue in chloroform was washed with 1 *N* hydrochloric acid, water, sodium bicarbonate solution, dried ($MgSO_4$), and evaporated to a solid, which was chromatographed on silica gel (2% MeOH in chloroform) and crystallized from ethyl acetate/petrol (3.24 g, 46%), mp 156–157°C, $[\alpha]_D^{20} = -188.7°$ (*c* 1, MeOH).

Anal. for $C_{17}H_{23}O_3N_3$: C, 64.3; H, 7.3; N, 13.2. Found: C, 64.4; H, 7.5; N, 13.2%.

Similar routes were used to prepare other proline-containing compounds (Table 6).

N-Benzyloxycarbonyl-L-alanine Ethylamide

N-Benzyloxycarbonyl-L-alanine (33.5 g, 0.15 *M*) in dry tetrahydrofuran (225 ml) was cooled to −10°C. Triethylamine (22.95 ml, 0.15 *M*) and *iso*butylchloroformate

TABLE 6

R.Ala.Pro.NHR[1]

R	R^1	mp	$[\alpha]_D^{20}$ (c = 1%)
CH_3CO-	$-CH_2CH_3$	140-3° (EtOAc)	-79.0° (DMF)
CH_3CH_2CO-	$-CH_2CH_3$	173-5° (EtOAc)	-63.9° (WATER)
$PhCH_2OCO$-	$-CH_2CH_3$	110-13° (EtOAc/Ether)	-96.0° (MeOH)
CH_3CH_2CO-	$-CH_3$	163-5° (EtOAc)	-137.0° (MeOH)
CH_3CH_2CO-	$-CH(CH_3)_2$	173-5° (EtOAc/Petrol)	-127.2° (MeOH)
CH_3CH_2CO-	$-(CH_2)_4CH_3$	105-8° (EtOAc/Petrol)	-151.7° (WATER)
CH_3CH_2CO-		172-3° (EtOAc)	-122.8° (MeOH)
CH_3CH_2CO-		172-4° (EtOAc)	-125.1° (MeOH)
CH_3CH_2CO-		155-6° (Ether/Petrol)	-119.4° (MeOH)
CH_3CH_2CO-		142-3° (Ether/Petrol)	-120.3° (MeOH)
CH_3CH_2CO-		169-71° (EtOAc)	-191.6° (MeOH)

R.Ala.Pro.NHR[1]

R	R^1	mp	$[\alpha]_D^{20}$ (c = 1%)
CH_3CH_2CO-		137-8° (EtOAc/Petrol)	-174.8° (MeOH)
CH_3CH_2CO-		204-5° (EtOAc/Petrol)	-151.8° (MeOH)
CH_3CH_2CO-	$-CH_2Ph$	-	-153.1° (WATER)
$(CH_3)_2CHCO$-	$-CH_2CH_3$	155-7° (EtOAc/Petrol)	-132.1° (MeOH)
$(CH_3)_3C.CO$-	$-CH_2CH_3$	-	-109.7° (MeOH)
$CH_3(CH_2)_3CO$-	$-CH_2CH_3$	110-111° (petrol)	-123.2° (MeOH)
CH_3CH_2OCO-	$-CH_2CH_3$	126-8° (petrol)	-117.6° (MeOH)
	$-CH_2CH_3$	191-2° (EtOAc)	-116.0° (MeOH)
PhCO-	$-CH_2CH_3$	200-202° (EtOAc)	-82.7° (MeOH)
$PhCH_2CO$-	$-CH_2CH_3$	127-30° (EtOAc/Petrol)	132.6° (MeOH)
Tos-	$-CH_2CH_3$	-	-90.7° (WATER)
Ac.Pro-	$-CH_2CH_3$	-	-
Ac.D.Ala-	$-CH_2CH_3$	163-5° (iPrOH/Ether)	-67.1° (MeOH)

R.Ala.Pro.NHR[1]

R	R^1	mp	$[\alpha]_D^{20}$ (c = 1%)
CH_3CO-	-Ph	163-5° (EtOAc/Petrol)	-186.2° (MeOH)
CF_3CO-	-Ph	204-7° (petrol)	-134.4° (AcOH)
PhCO-	-Ph	100-102° (EtOAc/Petrol)	-64.4° (MeOH)
$CH_3(CH_2)_4CO$-	-Ph	113-15° (EtOAc/Petrol)	-171.8° (MeOH)
$PhCH_2OCO$-	$-CH(CH_3)_2$	124-5° (EtOAc/Petrol)	-94.3° (MeOH)
$PhCH_2OCO$-		118-9° (Ether)	-93.5° (MeOH)
$PhCH_2OCO$-	$-(CH_2)_4CH_3$	103-5° (EtOAc/Petrol)	-94.6° (MeOH)

$PhCH_2OCO.X.Pro.NHR^1$

R	R^1	mp	$[\alpha]_D^{20}$ (c = 1%)
-Gly-	$-CH_2CH_3$	126-7° (EtOAc)	-75.0° (MeOH)
-Pro-	$-CH_2CH_3$	oil	-68.7° (MeOH)

(19.8 ml, 0.15 *M*) were added and the mixture stirred for 20 min at −10°C. A solution of ethylamine (7.53 g, 0.15 *M*) in dimethylformamide (215 ml) was added, the mixture was stirred at 0°C (1 hr), room temperature (16 hr), and evaporated. The residue in ethyl acetate (450 ml) was washed with 1 *N* hydrochloric acid, water, sodium bicarbonate solution, dried (Na_2SO_4), and evaporated. The oil crystallized from ethyl acetate/petrol (22.7 g, 61%), mp 127–128°C, $[\alpha]_D^{20} = +11.2$ (*c* 0.8, DMF).

L-Alanine-ethylamide Hydrobromide

The protected compound (10 g, 0.04 *M*) was dissolved in 4 *N* hydrogen bromide in acetic acid solution (50 ml) and after 1 hr dry ether was added. The product was filtered and dried *in vacuo* (8.0 g, 97%).

N-Benzyloxycarbonyl-L-alanyl-L-alanine Ethylamide

N-Benzyloxycarbonyl-L-alanine (7.5 g, 0.034 *M*) was coupled to L-alanine ethylamide using the mixed anhydride procedure to yield the product, which was

TABLE 7

R·ALA·ALA·NHR^1

R	R^1	mp (°C)	$[\alpha]_D^{20}$ (*c* = 1%)
CH_3CO–	$-CH_2CH_3$	267–268 (iPrOH)	−9.8° (DMF)
CH_3CO–	$-CH(CH_3)_2$	284–286 (EtOH)	−14.1° (DMF)
CH_3CO–	$-CH(CH_3)(C_2H_5)$	275–277 (iPrOH)	−79.2° (AcOH)
CH_3CH_2CO–	$-CH_2CH_3$	289–291 (EtOH)	−76.7° (MeOH)
CH_3CH_2CO–	–Ph	281–284 (MeOH)	−101.1° (AcOH)

recrystallized from dimethylformamide/water (9.05 g, 83%), mp 203–205°C, $[\alpha]_D^{20} = -1.1°$, $[\alpha]_{365}^{20} = -9.2$ (*c* 1, DMF).

Anal. for $C_{16}H_{23}O_4N_3O \cdot 5H_2O$: C, 58.2; H, 7.3; N, 12.7. Found: C, 58.2; H, 7.0; N, 12.7%.

This stepwise route, starting with *N*-benzyloxycarbonyl-L-alanine, was used to prepare the derivatives listed in Table 7.

Assay of Elastase Inhibition

The assay for elastase activity was based on the method of Visser and Blout (*36*) using *t*-Boc-L-Alanine-*p*-nitrophenyl ester as substrate. Inhibitors were added to pre-warmed buffered substrate as solutions in buffer or methanol (10–100 μl) and the reaction started by the addition of enzyme.

K_i was determined using a Dixon plot (*37*) either at two substrate concentrations or at one concentration using the intercept with $1/V$ which was determined by a Lineweaver–Burke plot (*38*).

Inhibition of Elastase-Induced Edema (in Collaboration with Dr. J. M. Hall)

Edema was induced with the left hind paw of female Sprague–Dawley rats (100–120 g) by the subcutaneous injection of 100 μg porcine pancreatic elastase (Sigma Type III) in 50 μl 1% aq. glycine and was measured after 2 hr by mercury displacement.

Drugs were administered orally at a dose of 100 mg/kg in 0.5% w/v tragacanth (20 ml/kg) 30 min prior to elastase injection. Drug effects were expressed as a percentage change in edema volume compared with untreated animals (elastase alone). Groups of six animals were used.

REFERENCES

1. M. C. GEOKAS, *Arch. Pathol.* **86**, 127 (1968).
2. A. JANOFF, *Ann. Rev. Med.* **23**, 177 (1972).
3. J. C. POWERS, *T.I.B.S.* 211 (1976).
4. "Pulmonary Emphysema and Proteolysis" (C. Mittmann, Ed.), pp. 1–537. Academic Press, New York, 1972.
5. H. C. WATSON, D. M. SHOTTON, J. M. COX, AND H. MUIRHEAD, *Nature (London)* **255**, 806 (1970); H. C. WATSON AND D. M. SHOTTON, *Nature (London)* **255**, 811 (1970).
6. D. M. SHOTTON AND B. S. HARTLEY, *Nature (London)* **255**, 802 (1970).
7. I. SCHECHTER AND A. BERGER, *Biochem. Biophys. Res. Commun.* **27**, 157 (1967).
8. R. C. THOMPSON AND E. R. BLOUT, *Proc. Natl. Acad. Sci. USA* **67**, 1734 (1970).
9. D. ATLAS, S. LEVIT, I. SCHECHTER, AND A. BERGER, *FEBS Lett.* **11**, 281 (1970).
10. D. ATLAS AND A. BERGER, *Biochemistry* **11**, 4719 (1972); **12**, 2577 (1973).
11. R. C. THOMPSON AND E. R. BLOUT, *Biochemistry* **12**, 44 (1973).
12. A. THOMSON AND I. S. DENNISS, *Eur. J. Biochem.* **38**, 1 (1973).
13. H. KAPLAN, V. B. SYMONDS, H. DUGAS, AND D. R. WHITAKER, *Canad. J. Biochem.* **48**, 649 (1970).
14. L. VISSER, D. S. SIGMAN, AND E. R. BLOUT, *Biochemistry* **10**, 735 (1971).
15. W. E. BROWN AND F. WOLD, *Biochemistry* **12**, 828, 835 (1973).
16. B. S. HARTLEY AND D. M. SHOTTON, "The Enzymes," 3rd ed., Vol. 3, pp. 323–366. Academic Press, New York, 1971.
17. J. C. POWERS AND P. M. TUHY, *Biochemistry* **12**, 4767 (1973).
18. L. DZIALOSZYNSKI AND T. HOFMANN, *Biochim. Biophys. Acta* **302**, 406 (1973).
19. C. P. DORN, M. ZIMMERMAN, S. S. YANG, E. C. YUREWICZ, B. M. ASHE, R. FRANKSHUN, AND H. JONES, *J. Med. Chem.* **20**, 1464 (1977).
20. R. C. THOMPSON AND E. R. BLOUT, *Biochemistry* **12**, 51 (1973).
21. R. C. THOMPSON AND E. R. BLOUT, *Biochemistry* **12**, 57 (1973).
22. R. C. THOMPSON, *Biochemistry* **12**, 47 (1973).
23. H. UMEZAWA, T. AOYAGI, A. OKURA, H. MORISHIMA, T. TAKEUCHI, AND Y. OKAMI, *J. Antibiot.* **26**, 787 (1973).
24. J. C. POWERS, B. F. GUPTON, A. D. HARLEY, N. NORIKAZU, AND R. J. WHITLEY, *Biochim. Biophys. Acta* **485**, 156 (1977).
25. J.-L. DIMICOLI, J. BIETH, AND J.-M. LHOSTE, *Biochemistry* **15**, 2230 (1976).
26. A. RENAUD, J.-L. DIMICOLI, P. LESTIENNE, AND J. BIETH, *J. Amer. Chem. Soc.* **100**, 1005 (1978).
27. J. C. POWERS AND D. L. CARROLL, *Biochem. Biophys. Res. Commun.* **67**, 639 (1975).
28. W. ARDELT, A. KOJ, J. CHUDZIK, AND A. DUBIN, *FEBS Lett.* **67**, 156 (1976).
29. R. E. SCOFIELD, R. P. WERNER, AND F. WOLD, *Biochemistry* **16**, 2492 (1977).
30. P. L. NAYAK AND M. L. BENDER, *Biochem. Biophys. Res. Commun.* **83**, 1178 (1978).
31. D. M. SHOTTON, N. J. WHITE, AND H. C. WATSON, *Cold Spring Harbor Symp. Quant. Biol.* **36**, 91 (1971).
32. D. ATLAS, *J. Mol. Biol.* **93**, 39 (1975).

33. L. SAWYER, D. M. SHOTTON, J. W. CAMPBELL, P. L. WENDELL, H. MUIRHEAD, H. C. WATSON, R. DIAMOND, AND R. C. LADNER, *J. Mol. Biol.* **118**, 137 (1978).
34. R. C. THOMPSON, *Biochemistry* **13**, 5495 (1974).
35. T. WIELAND, L. LAPATSANIS, J. FAESEL, AND W. KONZ, *Justus Liebigs Ann. Chem.* **747**, 194 (1971).
36. L. VISSER AND E. R. BLOUT, *Biochim. Biophys. Acta* **268**, 257 (1972).
37. M. DIXON, *Biochem. J.* **55**, 170 (1953).
38. H. LINEWEAVER AND D. BURKE, *J. Amer. Chem. Soc.* **56**, 658 (1934).
39. L. SAWYER AND H. C. WATSON, unpublished.